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Cloning, pharmacological characterization, and polymorphism screening of the guinea pig β_2-adrenoceptor

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Abstract

In asthma, β_2-adrenoceptor agonist responsiveness has been associated with Arg16Gly and Gln27Glu polymorphisms of the β_2-adrenoceptor. Since the guinea pig is extensively used as an animal model for asthma, we investigated the occurrence of possible polymorphism of the guinea pig β_2-adrenoceptor. The guinea pig β_2-adrenoceptor coding region was amplified by sequence homology-based cloning. Homology of the translated protein with the human β_2-adrenoceptor was 88% with Ala at position 16 and Glu at position 27. Radioligand binding and cAMP-accumulation experiments of Chinese hamster ovary (CHO) cells transfected with the guinea pig β_2-adrenoceptor revealed a homogeneous population of functional receptors. Five degenerate single nucleotide polymorphisms were found within the β_2-adrenoceptor coding region of outbred Dunkin Hartley guinea pigs, at residues 354, 453, 483, 534 and 642. In conclusion, we have cloned the guinea pig β_2-adrenoceptor, which shows to be functional upon expression in a recombinant system and contains five single nucleotide polymorphisms dissimilar to human polymorphisms.

Keywords: (Guinea pig); β_2-Adrenoceptor; Molecular cloning; Radioligand binding; cAMP; Single nucleotide polymorphism

1. Introduction

Reduced responsiveness to β_2-adrenoceptor agonists, either induced by allergen exposure or β_2-adrenoceptor agonist treatment (Jenne et al., 1977; Meurs et al., 1987; Meurs and Zaagsma, 1991; Barnes, 1995), has been found in patients suffering from allergic asthma. Nine polymorphisms have been described in the human β_2-adrenoceptor open reading frame. Four of these polymorphisms result in amino acid changes of the receptor, three of which are functionally important (Reihsaus et al., 1993). In recombinant Chinese hamster fibroblast (CHW-1102) cells, Thr164 substitution to Ile164 reduced β-adrenoceptor agonist binding and increased β-adrenoceptor agonist promoted down-regulation. However, the allelic frequency of this receptor variant represents only 2% in the Caucasian population (Green et al., 1993). The amino acid substitutions at amino acid position 16 (Arg16Gly) and 27 (Gln27Glu) have a higher population prevalence. Although neither Arg16Gly nor Gln27Glu polymorphism has been associated with the development of asthma (Reihsaus et al., 1993; Martinez et al., 1997; Dewar et al., 1997), several clinical studies have implicated a relationship between β_2-adrenoceptor polymorphisms at positions 16 and 27 and the severity of asthma-associated phenotypes, including airway hyperresponsiveness (Hall et al., 1995; Fowler et al., 2000), nocturnal asthma (Turki et al., 1995), and elevated IgE levels (Dewar et al., 1997). In addition, there is evidence for a relationship between β_2-adrenoceptor polymorphisms and reduced bronchodilating response to inhaled β_2-adrenocep-

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tor agonists (Martinez et al., 1997; Lima et al., 1999; Kotani et al., 1999). Moreover, the extent of desensitization of β_2-adrenoceptor agonist-induced bronchodilation or bronchoprotection against contractile stimuli seems to be related to the β_2-adrenoceptor genotype (Tan et al., 1997; Israel et al., 2000). Only few studies have addressed the role of Arg16Gly and Gln27Glu polymorphisms in the regulation of β_2-adrenoceptor function at a molecular or cellular level, with apparently conflicting results. Thus, in recombinant CHW-1102 cells, the Gly16 form of the receptor was associated with increased agonist-promoted downregulation, whereas the Glu27 form of the receptor appeared to protect against downregulation induced by β-adrenoceptor agonists (Green et al., 1994). In human airway smooth muscle cells, the Gly16 form of the β_2-adrenoceptor was also associated with enhanced agonist-induced downregulation, whereas the Glu27 form was associated with either decreased downregulation (Green et al., 1995) or increased desensitization of the β_2-adrenoceptor response (Moore et al., 2000). In human lung mast cells, both the Gly16 and Glu27 form of the receptor have been shown to protect against agonist-induced desensitization (Chong et al., 2000).

The guinea pig is being extensively used as an animal model for allergic asthma. Comparable to human asthmatics, allergen challenge of sensitized guinea pigs causes early and late asthmatic reactions, and subsequent airway hyperresponsiveness, accompanied by inflammation of the airways, i.e. increased numbers of eosinophils, T lymphocytes and neutrophils (Hutson et al., 1988; Frew et al., 1990; Boichot et al., 1991; Santing et al., 1994a). A variety of in vitro and in vivo studies have demonstrated both β-adrenoceptor agonist- and allergen-induced β_2-adrenoceptor desensitization of airway smooth muscle and inflammatory cells in this species (Meurs and Zaagsma, 1991; Douglas et al., 1977; Barnes et al., 1980; Santing et al., 1994b; Nishikawa et al., 1994; Wang et al., 1994; Mio et al., 2000). So far, the guinea pig β_2-adrenoceptor has not been cloned.

Several genetic studies in mammals (rodents and non-rodents) have shown mutations in coding regions, which are homologous to human mutations and possibly associated with hereditary disorders. For example, the Jacksons Laboratory's mouse genome database lists over 50 spontaneous mutations at the DNA level, which are similar to human gene mutations associated with a disease (O'Brien et al., 1999). Since β_2-adrenoceptors of different species show large homology with conserved presence of Gly at amino acid position 16 and Glu at position 27 (Dixon et al., 1986; Kobilka et al., 1987; Nakada et al., 1989; Buckland et al., 1990; Amend and Guan, 1995; Huang et al., 1997; Einspanier et al., 1999), we investigated whether functionally relevant polymorphisms in the guinea pig β_2-adrenoceptor could be detected.

We report the cloning, pharmacological characterization, and polymorphism screening of the outbred Dunkin Hartley guinea pig β_2-adrenoceptor.

2. Materials and methods

2.1. Reagents and pharmacological compounds

DNAzol® genomic DNA isolation reagent was purchased from the Molecular Research Centre (Cincinatti, OH, USA). Concentrated buffer for polymerase chain reaction (10 × PCR-buffer), deoxynucleotide triphosphate (dNTPs) and Taq-polymerase were from Invitek (Berlin, Germany). Primers were synthesized by GensetOligos (Paris, France). RNase/DNAs-free H_2O, 3-isobutyl-1-methyl xanthine (IBMX) and (−)-isoprenaline were purchased from Sigma (St. Louis, MO, USA). The pCR 2.1 TA-vector, PCR II-TOPO blunt-end vector and pcDNA3.1(−) expression vector were from Invitrogen (Breda, The Netherlands). The 3′ RACE-PCR kit, Dulbecco's Modified Eagle's Medium (DMEM-F12), fetal calf serum, L-glutamine, 5 × concentrated RT-buffer and M-MLV reverse transcriptase were purchased from Life Technologies (Breda, The Netherlands). Pwo-polymerase, restriction enzymes *Hin*dIII and *Bam*HI and random hexamers, used for reverse transcription were from Roche Diagnostics (Almere, The Netherlands). Saint transfection kit was purchased from Saint (Groningen, The Netherlands). The ThermoSequenase fluorescent primer cycle sequence kit was purchased from Amersham ('s Hertogenbosch, The Netherlands). G 418 was purchased from Calbiochem (La Jolla, CA, USA). Timolol was from Bufa (Uitgeest, The Netherlands). The radioactive compounds [^{3}H](−)-CGP12177A ((−)-4-(3-*t*-butylamino-2-hydroxypropoxy) benzimidazol-2-one) and [^{3}H]cAMP were purchased from Amersham and Du Pont-New England Nuclear (Beverly, MA, USA), respectively.

2.2. Animals and DNA extraction

Specified pathogen-free outbred Dunkin Hartly guinea pigs (Harlan, Heathfield, UK), weighing 400–500 g, were used in this study. The animals were housed in individual cages in climate-controlled animal quarters according to the University of Groningen Committee for Animal Experimentation, which is responsible for the care and proper use of experimental animals. Genomic DNA was extracted from homogenized guinea pig spleen using DNAzol®. Briefly, 50 mg tissue was homogenized in 1 ml DNAzol®. The homogenate was centrifuged for 10 min (10,000 × *g*). Genomic DNA was precipitated by addition of 0.5 ml 100% ethanol, washed twice with 75% ethanol and solubilized in 1 ml 8 mM NaOH; 117 μl 0.1 M HEPES was added to adjust the pH to 7.8.

2.3. Sequence homology-based cloning

All primers used for polymerase chain reaction (PCR) were designed, based on the interspecies homology between the sequences of hamster, human, mouse, rat, rhesus monkey, dog and bovine β_2-adrenoceptor genes, available in the

ENTREZ sequence database of the National Center of Biotechnology Information (accession numbers M15169, X03804, X17607, X15643, Z86037, L38905 and U73206, respectively). The sequence of the first forward primer was based on the interspecies homology of the 5′ promoter region and was located 31 bases upstream of the ATG start codon. Primers with the lowest probability to amplify any other gene were chosen.

PCR amplifications were carried out as described previously (Biber et al., 1997). In brief, the following reagents were added to 1 μg of guinea pig genomic DNA: 5 μl 10 × PCR-buffer, 2.5 μl 50 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 1 μl of each primer, 39 μl H_2O and 0.1 μl Taq-polymerase. Genomic DNA was amplified by 35 thermal cycles. Primer sequences and annealing temperatures are listed in Table 1. PCR products were size-fractioned on a 1.5% agarose gel. To amplify the 3′-end of the guinea pig β_2-adrenoceptor gene, a commercially available 3′ RACE-PCR kit was used. Briefly, total RNA was isolated from guinea pig cerebellum according to Chomczynski and Sacchi (1987) and transcribed into cDNA in a final volume of 20 μl containing 1 μg total RNA, 10 μl H_2O, 1 μl of an oligo-(dT) adapter antisense primer, 2 μl 10 × PCR-buffer, 2 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTPs, 2 μl 0.1 M dithiotreitol and 1 μl superscript II reverse transcriptase. After 50-min incubation at 42 °C, the reaction was terminated by heating at 70 °C for 15 min. Finally, 1 μl RNase H was added and the reaction was incubated at 37 °C for another 20 min. Subsequently, 1 μl of the reverse transcription-reaction was amplified by 35 thermal cycles as described above, using a gene specific sense primer (GSP1) and an antisense abridged universal amplification primer (AUAP). After size-fractioning, 2 μl of the PCR product was amplified by another 35 thermal cycles, using a second, nested gene specific sense primer (GSP2) to increase specificity of the amplified DNA fragment.

Table 1
Primers used for sequence homology-based cloning and 3′RACE-PCR

Primer	Annealing temperature (°C)	Nucleotide number[a]
5′-GCCGGTGCGCTCACCTGC-3′ (forward)	60	2228–2235
5′-CACGATGGCCAGGACGAT-3′ (reverse)	60	2378–2395
5′-ATCGTCCTGGCCATCGTG-3′ (forward)	62	2378–2395
5′-ATGAAGAAGGGCAGCCAGCA-3′ (reverse)	62	3104–3123
5′-TGCTGGCTGCCCTTCTTCAT-3′ (forward)	54	3104–3123
5′-GCTAGGCACAGTACCTTG-3′ (reverse)	54	3437–3454
5′-AACGATTGCTCCAGCAACAG-3′ (GSP1)	54	
5′-AGCCCAATGTTTGTCACCAG-3′ (GSP2)	54	

[a] The numbers correspond with the location of the nucleotides of the rat β_2-adrenoceptor open reading frame (accession number X17607, Entrez GenBank, NCBI); GSP = gene specific sense primer.

All amplified DNA fragments were subcloned into a pCR 2.1 TA-vector in a final volume of 10 μl containing both vector and PCR-product, 5 μl H_2O, 1 μl 10 × ligation buffer and 1 μl of T4 ligase. The reaction mixture was incubated at 14 °C for at least 18 h. Sequence analysis was performed on both strands. To exclude sequence errors produced by Taq-polymerase, sequence analysis was performed on at least six different clones.

2.4. Polymorphism screening

Genomic DNA extracted from spleen of 14 guinea pigs was amplified by 35 thermal cycles as described (annealing at 62 °C), using a proofreading Pwo-polymerase and the following ‘full-length’ primer pair: 5′-CGCTCACCTGC-TTCCCTTGC-3′ (forward) and 5′-TGTTCTGTTGAGGG-AAGGAAATCTT-3′ (reverse), starting 26 bases upstream and 20 bases downstream of the ATG startcodon and the TAA stopcodon, respectively. Both ‘full-length’ primer sequences were complementary to the DNA fragments obtained by homology-based cloning and 3′RACE-PCR. After size-fractioning, a DNA fragment of 1.3 kb, containing the complete β_2-adrenoceptor open reading frame, was subcloned into a PCR II-TOPO blunt-end vector in a final volume of 5 μl, containing 1 μl of the vector, 1 μl of the guinea pig β_2-adrenoceptor insert and 3 μl H_2O. The reaction was incubated at room temperature for 5 min and terminated by adding 1 μl 6 × TOPO stop solution. To detect polymorphisms, sequence analysis was performed on both strands of a single clone of 14 different animals. Sequence analysis was performed as described (Murray, 1989) using the ALF-sequence method and the ThermoSequenase fluorescent primer cycle sequence kit.

2.5. Vector construction

A *Hin*dIII/*Bam*HI fragment, containing the full-length guinea pig β_2-adrenoceptor coding region, was excised from the PCR II-TOPO blunt-end vector clone and ligated into a *Hin*dIII/*Bam*HI digested pcDNA3.1(−) expression vector, incorporating a neomycin resistance gene. Sequence analysis was performed to check the orientation of the guinea pig β_2-adrenoceptor insert.

2.6. Stable expression in Chinese hamster ovary (CHO) cells

CHO cells were cultured at 37 °C in incubation flasks, containing 10 ml DMEM-F12 medium, supplemented with 10% fetal calf serum and 2 mM L-glutamine in a humidified atmosphere (5% CO_2). Cells were seeded into six-well dishes and transfected with 1 μg of the pcDNA3.1(−)-guinea pig β_2-adrenoceptor expression vector, using 30 μl

Saint transfection kit. After 2 weeks of selection with 50 μg/ml G 418, monoclonal CHO cell lines, stably expressing the guinea pig β_2-adrenoceptor (CHO-Gpβ_2), were generated and guinea pig β_2-adrenoceptor mRNA expression levels of 10 CHO-Gpβ_2 cell lines were determined by RT-PCR. Briefly, 1 μg total CHO-Gpβ_2 RNA was transcribed into cDNA in a final volume of 25 μl, containing 1 μl 2.5 mM random hexamers, 5 μl 5 × RT-buffer, 5 μl 2.5 mM dNTPs, 0.5 μl RNase inhibitor and 0.5 μl M-MLV reverse transcriptase. After 15 min of incubation at 65 °C, 5 min at 4 °C and 60 min at 42 °C, the reaction was stopped by heating at 95 °C for 5 min. cDNA was amplified by 28 thermal cycles, using primer pairs for both the guinea pig β_2-adrenoceptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for GAPDH were as follows: 5′-CATCCTGCACCACCAACTGCTTAG-3′ (forward) and 5′-GCCTGCTTCACCACCTTCTTGATG-3′ (reverse). PCR-products were size-fractioned and β_2-adrenoceptor mRNA expression was determined relative to GAPDH mRNA expression. The CHO-Gpβ_2 cell line with the highest β_2-adrenoceptor mRNA expression was used for both radioligand binding and cAMP-accumulation assays.

2.7. Radioligand binding assay

CHO-Gpβ_2 cells were seeded in six-well dishes at 3×10^5 cells/well, 48 h prior to radioligand incubation. DMEM-F12 medium was replaced by incubation buffer (118 mM NaCl; 4.7 mM KCl; 3 mM $CaCl_2$; 1.2 mM $MgSO_4$; 0.5 mM EDTA; 10 mM glucose; 20 mM HEPES; pH 7.4) and cells were washed three times. For saturation binding experiments, intact CHO-Gpβ_2 cells were incubated overnight at 4 °C in 1 ml buffer, containing different concentrations of [^{3}H](−)-CGP12177 (45 Ci/mmol) ranging from 0.05 to 10 nM, in the absence or presence of timolol (1 μM) to define nonspecific binding. For competition experiments, cells were incubated overnight with [^{3}H](−)-CGP12177 (0.5 nM) at 4 °C in 1 ml buffer in the absence or presence of different concentrations of timolol, ranging from 1 pM to 1 μM. To stop the reaction,

```
1     ATG GGA CAC CTT GGG AAC GGC AGC GAC TTC TTG TTG GCA CCC AAC  45
      M   G   H   L   G   N   G   S   D   F   L   L   A   P   N
46    GCG AGC CAC GCG CCG GAC CAC AAC GTC ACG CGG GAA CGG GAT GAG  90
      A   S   H   A   P   D   H   N   V   T   R   E   R   D   E
91    GCT TGG GTG GTG GGC ATG GCC ATC GTC ATG TCG CTC ATC GTC CTG  135
      A   W   V   V   G   M   A   I   V   M   S   L   I   V   L
136   GCC ATC GTG TTC GGC AAC GTG CTG GTC ATC ACA GCC ATT GCC AAG  180
      A   I   V   F   G   N   V   L   V   I   T   A   I   A   K
181   TTT GAA CGA CTG CAA ACG GTC ACC AAC TAC TTC ATC ACC TCG CTG  225
      F   E   R   L   Q   T   V   T   N   Y   F   I   T   S   L
226   GCC TGT GCT GAC CTA GTC ATG GGC CTA GCG GTG GTG CCA TTT GGG  270
      A   C   A   D   L   V   M   G   L   A   V   V   P   F   G
271   GCC AGC CAC ATC CTC ATG AAC ATG TGG ACT TTT GGC AAC TTC TGG  315
      A   S   H   I   L   M   N   M   W   T   F   G   N   F   W
316   TGT GAG TTT TGG ACT TCC ATT GAT GTG CTG TGC GTC ACT GCC AGC  360
      C   E   F   W   T   S   I   D   V   L   C   V   T   A   S
361   ATT GAG ACC CTG TGC GTG ATC GCA GTG GAT CGA TAC TTC GCC ATC  405
      I   E   T   L   C   V   I   A   V   D   R   Y   F   A   I
406   ACG TCG CCT TTC AAG TAC CAG AGC CTA CTG ACC AAG AAT AAG GCT  450
      T   S   P   F   K   Y   Q   S   L   L   T   K   N   K   A
451   CGG GTG GTC ATC TTG ATG GTA TGG GTT GTG TCG GGC CTT ACA TCC  495
      R   V   V   I   L   M   V   W   V   V   S   G   L   T   S
496   TTC TTG CCC ATT CAG ATG CAC TGG TAC CGG GCC ACC CAC AAG GAC  540
      F   L   P   I   Q   M   H   W   Y   R   A   T   H   K   D
541   GCC ATC AAC TGC TAT GCG GAG GAG ACC TGT TGT GAC TTC TTC ACG  585
      A   I   N   C   Y   A   E   E   T   C   C   D   F   F   T
586   AAC CAA GCC TAT GCC ATT GCC TCC TCC ATC GTG TCC TTC TAC TTA  630
      N   Q   A   Y   A   I   A   S   S   I   V   S   F   Y   L
631   CCC TTG GTG GTT ATG GTC TTT GTC TAC TCC AGG GTC TTC CAG GTG  675
      P   L   V   V   M   V   F   V   Y   S   R   V   F   Q   V
676   GCC AAA AAG CAG CTC CAG AAG ATT GAC AGA TCT GAG GGC CGA TTC  720
      A   K   K   Q   L   Q   K   I   D   R   S   E   G   R   F
721   CAC ACC CAA AAC CTC AGC CAG GTG GAG CAG GAT GGG CGG AGT GGA  765
      H   T   Q   N   L   S   Q   V   E   Q   D   G   R   S   G
766   CAT GGA CTT CGC AGG TCC TCC AAG TTC TAC TTG AAA GAA CAC AAA  810
      H   G   L   R   R   S   S   K   F   Y   L   K   E   H   K
811   GCC CTC AAG ACC TTG GGC ATC ATC ATG GGC ACT TTC ACC CTC TGC  855
      A   L   K   T   L   G   I   I   M   G   T   F   T   L   C
856   TGG CTG CCC TTC TTC ATC GTC AAC ATT GTG CAC GTG ATT CAG GAC  900
      W   L   P   F   F   I   V   N   I   V   H   V   I   Q   D
901   AAC CTC ATC CCC AAG GAG GTG TAC ATC CTG CTG AAC TGG GTG GGC  945
      N   L   I   P   K   E   V   Y   I   L   L   N   W   V   G
946   TAT GTC AAT TCT GCT TTT AAC CCC CTC ATC TAC TGC CGG AGC CCA  990
      Y   V   N   S   A   F   N   P   L   I   Y   C   R   S   P
991   GAT TTC AGG ATT GCT TTC CAG GAG CTA CTG TGT CTT CGC AGA TCT  1035
      D   F   R   I   A   F   Q   E   L   L   C   L   R   R   S
1036  GCT TTG AAG GCT TAT GGG AAC GAT TGC TCC AGC AAC AGC AAC GGC  1080
      A   L   K   A   Y   G   N   D   C   S   S   N   S   N   G
1081  AAA ACG GAC TAC ACC GGG GAG CCC AAT GTT TGT CAC CAG GGG CAG  1125
      K   T   D   Y   T   G   E   P   N   V   C   H   Q   G   Q
1126  GAG AAA GAG AGG GAA CTG CTG TGT GAG GAC CCC CCG GGC ACA GAA  1170
      E   K   E   R   E   L   L   C   E   D   P   P   G   T   E
1171  GAC TTG GTG AGC TGT CCA GGT ACT GTG CCT AGT GAT AGC ATT GAT  1215
      D   L   V   S   C   P   G   T   V   P   S   D   S   I   D
1216  TCA CAA GGG AGG AAC TAT AGT ACA AAT GAC TCA CTG CTC TAA      1257
      S   Q   G   R   N   Y   S   T   N   D   S   L   L   *
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Fig. 1. Nucleotide sequence and translated amino acid sequence of the guinea pig β_2-adrenoceptor (accession number AJ459814). The ATG startcodon is shown in boldface. The single nucleotide polymorphisms at positions 354 (t/c), 453 (g/a), 483 (g/a), 534 (c/t) and 642 (t/a) are shaded.

cells were washed three times with 2 ml ice-cold buffer and cells were lysed in 1 ml NaOH (0.5 M). The cell extract was mixed with 4 ml scintillation fluid and [^{3}H](−)-CGP12177 binding was quantified by scintillation counting.

2.8. cAMP-accumulation assay

CHO-Gpβ_2 cells were seeded in 24-well plates at 1.5×10^5 cells/well 48 h prior to stimulation. DMEM-F12

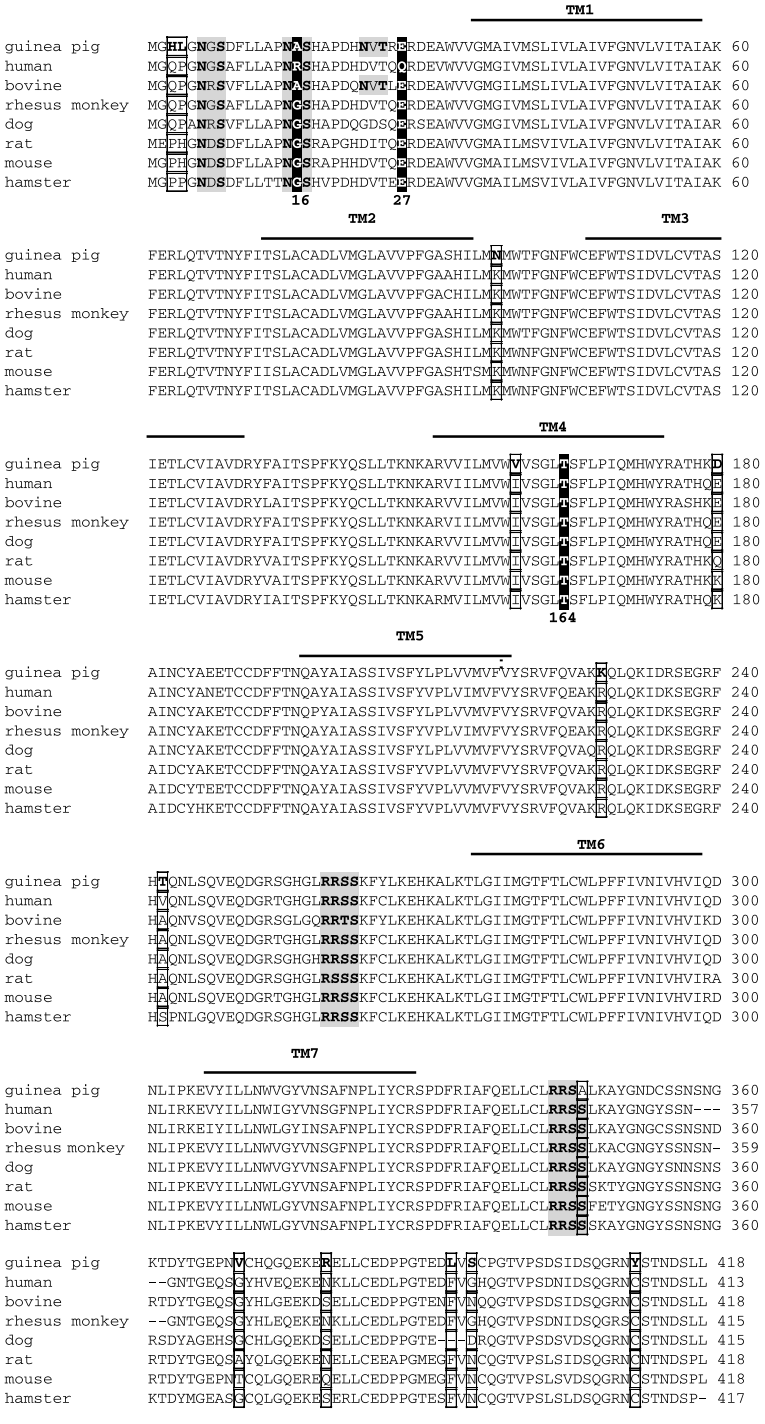

Fig. 2. Comparison of several mammalian β_2-adrenoceptor amino acid sequences. The guinea pig β_2-adrenoceptor protein sequence is compared to the β_2-adrenoceptor sequence of human, bovine, rhesus monkey, dog, rat, mouse and hamster, respectively. The seven transmembrane spanning domains are indicated by the horizontal lines (TM1 – TM7). Human polymorphic amino acid positions (16, 27 and 164) are shown in black. Signal sequences for N-linked glycosylation (-N-X-S/T-) and consensus sequences for PKA phosphorylation (-RRSS-) are darkly shaded. Residues only found in the guinea pig β_2-adrenoceptor are shown in boxes.

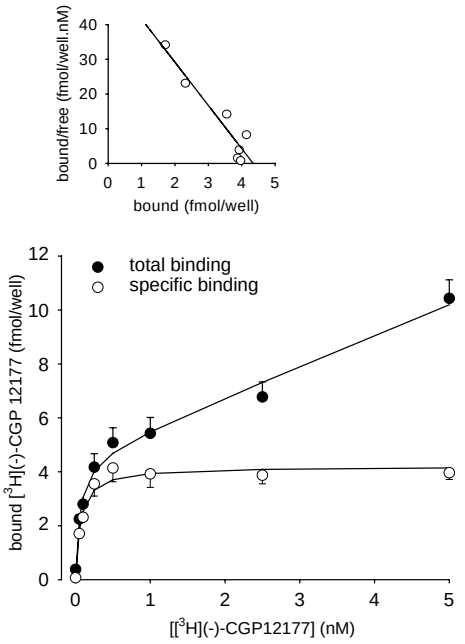

Fig. 3. [^{3}H](−)-CGP12177 saturation binding to the guinea pig β_2-adrenoceptor expressed in CHO cells, with 1.5×10^6 cells per well. Data represent mean values of four experiments. Nonspecific binding was determined in the presence of 1 μM timolol. The inset shows a Scatchard plot of the average specific binding data ($r = 0.95$).

medium was replaced by 180 μl stimulation buffer (118 mM NaCl; 4.7 mM KCl; 3 mM $CaCl_2$; 1.2 mM $MgSO_4$; 0.5 mM EDTA; 10 mM glucose; 20 mM HEPES; pH 7.4), supplemented with IBMX (0.1 mM) and cells were incubated at 37 °C for 6 min with different concentrations of (−)-isoprenaline, ranging from 0.1 nM to 1 mM, in the absence or presence of timolol (100 nM). Reactions were stopped immediately with 200 μl ice-cold 3.5% perchloric acid and placed on ice for at least 30 min. Samples were neutralized with 100 μl 50% saturated $KHCO_3$ and a competitive protein binding assay was used to determine cAMP levels as described earlier (Sipma et al., 1996). Briefly, 50 μl sample was incubated with 200 μl buffer containing 50 mM Tris–HCl, 4 mM EDTA, 160 μg cAMP binding protein, 1 mg bovine serum albumin and 190 nM [^{3}H]cAMP (30 Ci/mmol) at 4 °C for at least 2 h. The reaction was terminated by adding 1000 μl charcoal suspension (Norit A special, 3.5 g/l) followed by centrifugation at 3000 rpm for 15 min to remove the excess of unbound [^{3}H]cAMP. Radioactivity in the supernatant was measured by scintillation counting.

2.9. Data analysis

All data are expressed as the mean ± S.E.M. of four to six experiments. K_d and B_{max} values for [^{3}H](−)-CGP12177 were determined by Scatchard analysis. The pK_i value for timolol was calculated according to the equation: $pK_i = -\log [IC_{50}/(1+[C]/K_d)]$, with $[C]$ being the concentration of [^{3}H](−)-CGP12177, used in the competition binding assay. The pK_B value for timolol was calculated according to the equation: $pK_B = -\log [B] + \log [(EC_{50B}/EC_{50}) - 1]$, with $[B]$ being the concentration of timolol and EC_{50B} the EC_{50} of (−)-isoprenaline for cAMP- accumulation in the presence of timolol.

3. Results

3.1. Cloning strategy and sequence analysis

Three DNA fragments of 174, 352 and 747 base pairs (bp), respectively, were amplified, using different homology-based primer subsets (Table 1). Rat genomic DNA served as a positive control. 3′RACE-PCR with GSP1 resulted in a DNA fragment of approximately 650 bp. The second, nested gene specific primer (GSP2) reduced the DNA fragment to approximately 500 bp. PCR with a full-length primer pair resulted in a DNA fragment of 1325 bp.

Subsequent cloning and sequence analysis revealed an intronless open reading frame of 1257 bp, coding for a protein very similar to the mammalian β_2-adrenoceptor (Fig. 1). A comparison of the translated guinea pig β_2-adrenoceptor open reading frame with previously reported β_2-adrenoceptors from human, rhesus monkey, bovine, dog, rat, mouse and hamster, respectively, shows the largest homology with rat (89%) and mouse (90%), with the highest similarity in the hydrophobic transmembrane segments (Fig. 2). The overall homology with the human β_2-adrenoceptor coding region is 88%.

The amino terminal extracellular domain of the guinea pig β_2-adrenoceptor contains two highly conserved consensus signal sequences (-N-G-S-; -N-A-S-) for N-linked glycosylation as well as one putative signal sequence (-N-V-T-), which is also found in bovine. As part of the second conserved glycosylation site, at position 16, both bovine

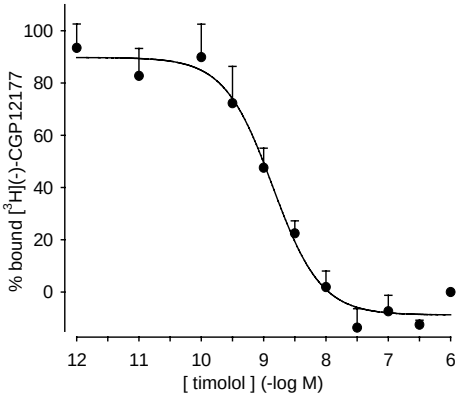

Fig. 4. Inhibition of [^{3}H](−)-CGP12177 binding to CHO-Gpβ_2 cells by timolol. [^{3}H](−)-CGP12177 binding is expressed as a percentage of specific binding obtained in the presence of 0.5 nM of the radioligand. Data represent mean values ± S.E.M. from four separate experiments performed in duplicate.

and guinea pig contain an Ala instead of a Gly. In human, Arg16Gly polymorphism is found at this position. Like all other mammalian β_2-adrenoceptors, the guinea pig β_2-adrenoceptor contains a Glu at the human polymorphic Gln27Glu and a Thr at the human polymorphic Thr164Ile amino acid position. In the guinea pig, an Ala is found at position 346 of the intracellular carboxy terminal domain, whereas all other species share a serine at this position, as part of a highly conserved phosphorylation site (-R-R-S-S-) for protein kinase A (PKA).

3.2. Pharmacological characterization

To study its pharmacological properties, the guinea pig β_2-adrenoceptor open reading frame was subcloned into the pcDNA3.1(−) vector and stably expressed in CHO cells. Saturation binding studies were performed in intact CHO-Gpβ_2 cells, using the hydrophilic ligand [^{3}H](−)-CGP12177 (Fig. 3). Scatchard plot analysis revealed a single population of binding sites with a B_{max} of 4.53 ± 0.47 fmol/well, corresponding to 3.02 ± 0.31 fmol/10^6 cells and 1817 ± 191 receptors/cell. A K_d of 0.10 ± 0.02 nM was obtained. The nonselective β-adrenoceptor antagonist timolol induced a monophasic inhibition of [^{3}H](−)-CGP12177 binding in intact CHO-Gpβ_2 cells (Fig. 4), resulting in a pK_i for timolol of 9.82 ± 0.10.

Intact CHO-Gpβ_2 cells were stimulated with increasing concentrations of (−)-isoprenaline. In the presence of 0.1 mM IBMX, (−)-isoprenaline caused a concentration-dependent accumulation of cAMP, with a maximal effect (E_{max}) of 74.7 ± 6.6 pmol/well and a pEC_{50} of 7.92 ± 0.17. Timolol (100 nM) caused a parallel shift to the right of the (−)-isoprenaline concentration–response curve of 2.5 log units (Fig. 5), indicating competitive antagonism at the receptor, with a pK_B of 9.83 ± 0.19.

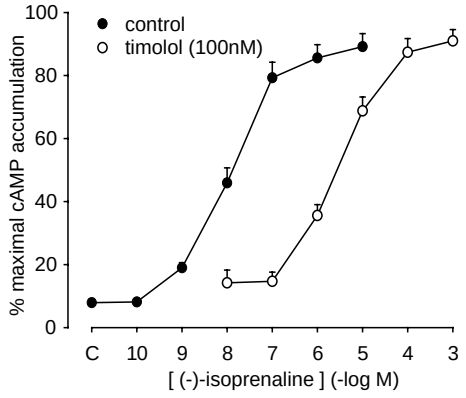

Fig. 5. (−)-Isoprenaline-induced cAMP-accumulation of CHO-Gpβ_2 cells in the absence (control) or presence of timolol (100 nM). cAMP-accumulation was measured in the presence of 0.1 mM IBMX and normalized to the maximal effect (E_{max}) of (−)-isoprenaline in each individual experiment. Data represent means ± S.E.M. from five to six separate experiments performed in duplicate.

3.3. Screening of polymorphisms

The β_2-adrenoceptor open reading frame of 14 male outbred Dunkin Hartley guinea pigs was screened for single nucleotide polymorphisms. Five polymorphisms were found at nucleic acid residues 354 (t/c), 453 (g/a), 483 (g/a), 534 (c/t) and 642 (t/a), respectively (Fig. 1). The mutations, within the triplet codons for amino acids 118, 151, 161, 178 and 213, occurred with a frequency varying between 28% and 48%. However, all of the single nucleotide polymorphisms were degenerate, i.e. not leading to a change in the encoded amino acids. Moreover, they did not show any similarity with polymorphisms found in the human β_2-adrenoceptor coding region.

4. Discussion

There exists a significant association between several polymorphisms of the β_2-adrenoceptor and asthma-associated phenotypes as well as β_2-adrenoceptor agonist responsiveness in human asthmatics (Reihsaus et al., 1993; Martinez et al., 1997; Dewar et al., 1997; Hall et al., 1995; Fowler et al., 2000; Turki et al., 1995; Lima et al., 1999; Kotani et al., 1999; Tan et al., 1997; Israel et al., 2000). Since the experimental asthma phenotype of the guinea pig is strikingly similar to human asthma, we investigated if polymorphisms, homologous to those of the human β_2-adrenoceptor, are present in the guinea pig β_2-adrenoceptor. Searching for polymorphisms in animal models may be of great help in understanding the genetic background of several diseases (O'Brien et al., 1999). This is the first study screening for single nucleotide polymorphisms in an outbred guinea pig strain, which constitutes a good animal model for allergic asthma (Hutson et al., 1988; Frew et al., 1990; Boichot et al., 1991; Santing et al., 1994a).

In the present study, the guinea pig β_2-adrenoceptor has been cloned and the receptor was functional in a recombinant system. Radioligand binding with [^{3}H](−)-CGP12177 was saturable, and yielded a K_d value for the radioligand of 0.10 nM. The affinity of [^{3}H](−)-CGP12177 for the guinea pig β_2-adrenoceptor was slightly higher than for the human β_2-adrenoceptor expressed in CHO cells (Pauwels et al., 1991). Stimulation of the CHO-Gpβ_2 cells with (−)-isoprenaline caused a concentration-dependent accumulation of cAMP, which was competitively antagonized by the nonselective β-adrenoceptor antagonist timolol. A pK_B value of 9.83 was found, which is comparable to the pK_B value of timolol against (−)-adrenaline-induced positive inotropy in human atria (Wang et al., 1996). As may be expected, the pK_B value of timolol against (−)-isoprenaline induced cAMP-accumulation was similar to its pK_i value of radioligand receptor binding.

The overall homology of the guinea pig β_2-adrenoceptor with the human β_2-adrenoceptor is 88%. The guinea pig β_2-

adrenoceptor contains a Glu at the human polymorphic Gln27Glu and a Thr at the human polymorphic Thr164Ile amino acid position. When compared to previously reported mammalian β_2-adrenoceptors (Dixon et al., 1986; Kobilka et al., 1987; Nakada et al., 1989; Buckland et al., 1990; Amend and Guan, 1995; Huang et al., 1997; Einspanier et al., 1999), there are 13 amino acids exclusively present in the guinea pig β_2-adrenoceptor. Sequence analysis of 14 animals revealed five single nucleotide polymorphisms in the β_2-adrenoceptor open reading frame. However, these polymorphisms are degenerate and dissimilar to polymorphisms found in the human β_2-adrenoceptor. Moreover, at the human polymorphic Arg16Gly amino acid position, the guinea pig β_2-adrenoceptor contains an Ala instead of Gly, as has also been observed in the bovine β_2-adrenoceptor (Einspanier et al., 1999). It cannot be excluded that investigating more guinea pigs might reveal other single nucleotide polymorphisms. In view of the relative high population prevalence of the single nucleotide polymorphisms at positions 16 and 27 of the human β_2-adrenoceptor, our results indicate that the Dunkin Hartley guinea pig strain is less suitable as a model to investigate the role of these common human β_2-adrenoceptor polymorphisms in allergic asthma.

In addition to the above-mentioned dissimilarities, the guinea pig β_2-adrenoceptor coding region bears a few interesting unique differences. Mammalian β_2-adrenoceptors generally contain two PKA consensus sites, located in the third intracellular loop ($RRSS^{259-262}$) and the carboxy terminal intracellular tail ($RRSS^{343-346}$) (Hausdorff et al., 1990; Clark et al., 1989). Unlike all other cloned β_2-adrenoceptors, the guinea pig β_2-adrenoceptor contains an Ala at position 346 instead of Ser, possibly implicating that the guinea pig β_2-adrenoceptor is less susceptible to agonist-induced desensitization via phosphorylation by PKA (Moffett et al., 1996). Mammalian β_2-adrenoceptors also usually contain two signal sequences for N-linked glycosylation (N-X-S/T-), where X represents any amino acid except a proline (Raymond et al., 1990). N-linked glycosylation appears to be essential for correct receptor trafficking to the cell surface and stability in the cell membrane (Rands et al., 1990). In addition to the two highly conserved glycosylation signal sequences, both the guinea pig and bovine β_2-adrenoceptor contain a putative third sequence (-N-X-T-) for N-linked glycosylation in the amino extracellular tail, which could possibly influence their membrane expression and rate of agonist-induced downregulation. This hypothesis is supported by a recent study, demonstrating that increased N-linked glycosylation of the β_1-adrenoceptor is associated with attenuated agonist-promoted downregulation (Rathz et al., 2002).

In conclusion, we have cloned the guinea pig β_2-adrenoceptor, which shows to be functional upon expression in CHO cells. Cloning of this receptor is important because it enables to compare its pharmacological properties with the human ortholog in the same recombinant system. Sequence analysis of 14 outbred Dunkin Hartley guinea pigs revealed five degenerate single nucleotide polymorphisms in the β_2-adrenoceptor coding region, which are dissimilar to polymorphisms found in the human β_2-adrenoceptor gene. Although the guinea pig has proven to be an excellent animal model for the investigation of several features of allergic asthma, the outbred Dunkin Hartley guinea pig strain appears less suitable as an animal model to elucidate the function of the human β_2-adrenoceptor polymorphisms at positions 16 and 27. The possible consequences of the absence of a serine in the consensus site for PKA phosphorylation and the presence of a putative third signal sequence for N-linked glycosylation for guinea pig β_2-adrenoceptor function remain to be established.

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